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A *Drosophila melanogaster hobo-white*$^+$ vector mediates low frequency gene transfer in *D. virilis* with full interspecific *white*$^+$ complementation

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Abstract

Transformation of a *Drosophila virilis white* mutant host strain was attempted using a *hobo* vector containing the *D. melanogaster* mini-*white*$^+$ cassette (*H*[*w*$^+$, hawN]) and an unmodified or heat shock regulated *hobo* transposase helper. Two transformant lines were recovered with the unmodified helper (HFL1), one containing only the *white*$^+$ marked vector, and a sibling line containing the vector as well as an HFL1 helper integration. An approximate total transformation frequency of 1% is deduced. A high frequency of wing and eye morphology mutants were also observed, suggesting that *hobo* may have mobilized a related element in *D. virilis*. The data reaffirms a relatively low transformation vector activity for the *hobo* transposon in *D. virilis*; however, nearly full interspecific expression of the *white*$^+$ marker supports its possible function in other species as well.

Keywords: germline transformation, *hobo* vector, transposable elements, white-eye gene, *Drosophila virilis*.

Introduction

Since the advent of routine *P*-element mediated germline transformation of *Drosophila melanogaster* (Rubin & Spradling, 1982), a primary aim of basic and applied molecular entomologists has been the analogous germline transformation of agriculturally and medically important insects (see Handler & O'Brochta, 1991). The testing of *P* vectors in nondrosophilid insects, however, has not resulted in successful gene transfer (Handler & O'Brochta, 1991) and transient excision assays have indicated restrictions in *P* function in all nondrosophilid insects tested (Handler *et al.*, 1993). Recently, gene transfer has been reported in medfly mediated by the *D. hydei Minos* element (Loukeris *et al.*, 1995) as well as *hobo*-mediated transformation of *D. virilis* (Lozovskaya *et al.*, 1996), with both occurring at or below a frequency of 1% of fertile G0s. In contrast, transformation of *D. melanogaster* with the *Musca domestica Hermes* element (O'Brochta *et al.*, 1996) has been reported at frequencies similar to, if not greater than, *hobo* transformation of *D. melanogaster* (Blackman *et al.*, 1989).

Thus, despite the fact that *hobo* and *Hermes* share considerable structural similarity (Warren *et al.*, 1994), based on germline transformation, *Hermes* is much more active in *D. melanogaster* than *hobo* is in *D. virilis*. The relatively low level of *hobo* vector function is notable, considering the much closer evolutionary distance between the source of the vector and the target species, relative to *Hermes* and *D. melanogaster*. The low level of *D. virilis hobo* transformation is also unexpected, due to results from transient embryonic *hobo* excision assays. We found that in the presence of *hobo* transposase, precise *hobo* excision occurred at similar frequencies with the same types of excision products in both *D. melanogaster* and *D. virilis* (Handler & Gomez, 1995), whereas precise excision occurred rarely in more distant tephritid species (Handler & Gomez, 1996). This suggested that *hobo*-mediated transformation of *D. virilis* might occur at frequencies approaching that in *D. melanogaster*.

Possible explanations for these inconsistencies are that *hobo* transposition is somehow restricted in *D. virilis*, or that an unrepresentative low transformation frequency in the single *D. virilis* experiment occurred, possibly caused by limitations specific to the helper or vector plasmids used. Restrictions on chromosomal transposition may result from considerably higher repetitive DNA content in *D. virilis* compared to *D.*

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melanogaster (Gall & Atherton, 1974) acting to restrict chromosomal insertions and/or function of the marker gene. Indeed, given that the *D. melanogaster* wild-type *white*$^+$ gene was used as a marker and strong red-eye expression only occurred in transformants having three independent insertions (Lozovskaya *et al.*, 1996), it is possible that full interspecific *white*$^+$ complementation cannot occur, or that position effect variegation suppressing *white*$^+$ expression in transformants (Hazelrigg *et al.*, 1984; Pirotta *et al.*, 1985) is much more prevalent in *D. virilis*. The medfly, *Ceratitis capitata*, also has a relatively high repetitive DNA content which may have similarly restricted *Minos* transformation or detection (Loukeris *et al.*, 1995).

To further explore the function of the *D. melanogaster hobo* gene transfer vector and *white*$^+$ marker in *D. virilis*, we have repeated transformation in this species utilizing a *hobo-white*$^+$ vector, and an unmodified HFL1 *hobo* transposase helper, as well the *hsp*70-regulated helper (Calvi & Gelbart, 1994). We report that whereas the *D. melanogaster white*$^+$ gene is capable of nearly normal function in *D. virilis*, *hobo* transformation occurred at a rate consistent with that previously reported by Lozovskaya *et al.* (1996).

Results

Transformant isolation

Approximately 600 *D. virilis white*$^{50-112}$ (w^-; mutant white eye) embryos were injected with the vector *H*[w^+, hawN] and HFL1 helper plasmid, having *hobo* transposase under normal promoter regulation, and 480 embryos injected with the same vector and the HSH2 heat-shock-regulated helper. From the HSH2 helper experiment, 135 G0 adults emerged which were individually outcrossed to two w^- adults, yielding a G0 fertility rate of 86%. All G0s and G1 offspring were examined for red-eye coloration which was not detectable in any of the flies.

In the HFL1 helper experiment 265 G0 adults emerged including 121 females and 144 males. Forty-two lines were created by intermating three female to two male G0s, with an additional thirty lines mating two G0 males to four wild type females. One additional line (no. 73) was created by the intermating of four G0 females and three G0 males, all of which had a curved-wing phenotype. None of the G0s showed evidence of eye pigmentation. Of the seventy-three lines, five red-eye G1 offspring were observed only in the curved-wing G0 line. Close visual inspection revealed a nearly identical red-eye coloration in the transformants relative to wild type (Fig. 1). The five G1 flies were individually outcrossed to w^- mutant flies, with the resulting G2 red-eye offspring inbred (sublines designated as R1–R5). One of the G1 (R3) females was sterile. Due to intermating, the precise number of fertile G0s could not be determined, though in the HSH2 helper experiment and previous experiments with *D. virilis* we consistently obtained an approximate fertility rate of 70–80%. Since hybridization tests indicated the same single event (see below) in the four G1s from line 73 (designated as *Dv*[*hawN*]*G73*), we estimate an approximate 0.5% frequency of transformation based on *white*$^+$ marker selection.

Sex-linkage for the four red-eye lines was determined by performing single pair matings between G2 red-eye males and white-eye females. The G3 female offspring of all these matings were red-eye, whereas the males were all white-eye indicating linkage to the X chromosome. Since the w^+ gene is normally sex-linked in *D. virilis*, at this point the possibility existed that the putative transformant was actually a *w* reversion.

Non-vector related phenotypes

A unilateral eyeless or shrunken eye phenotype was observed in three G0 lines, though all the G1s died within 3–5 days of emergence without reproducing. As noted above seven G0s had curved-wing phenotypes, and thirteen other G0 lines gave rise to G1 individuals with curved wings or shrivelled wings, with only one G1 curved wing arising from the G0 *Dv*[*hawN*]*G73* line. In subsequent generations all four *Dv*[*hawN*]*G73* sublines gave rise to curved- and shrivelled-wing flies (Fig. 2), though they were predominant in lines R1 and R2. We believe the wing phenotypes to be variations of the same genotype since inbreeding of each phenotype gave rise to both phenotypes as well as wild type. Although this indicated that the mutation is not a simple recessive, low ratios of mutants to wild type do not support it being dominant unless it is semi-lethal dominant as well. Pure-bred lines and determination of specific linkages have not been possible, though they occur in non-transformed white-eye siblings of *Dv*[*hawN*]*G73* and are not linked to the *H*[w^{++}, hawN] integration.

Hybridization analysis

Southern hybridization was performed on genomic DNA samples from wild-type and white-eye mutant host flies, curved-wing flies, and putative transformant straight-winged red-eye flies in the four sublines, in addition to white-eye siblings. DNA was first digested with *Eco*RI which releases the 4.2 kb mini-*white* gene from the *hobo* vector, and hybridized to probe containing the 2.2 kb *Pvu*I fragment of *H*[w^{++}, hawN], which overlaps 1.4 kb of the mini-*white* gene and 0.8 kb of

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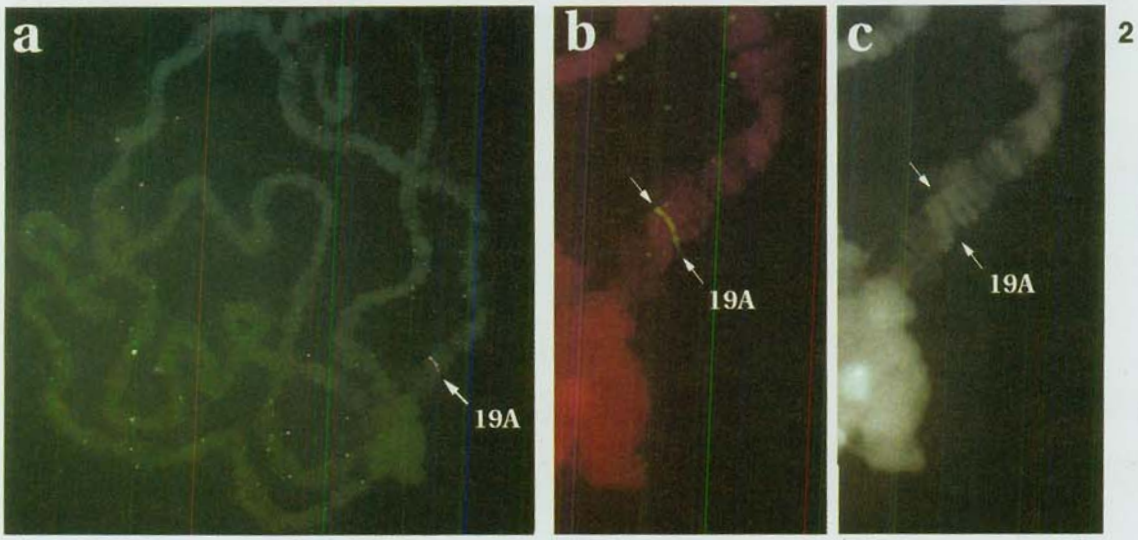

Figure 1. Eye colour phenotypes of the *D. virilis white*^{50112} mutant host (left), a *Dv*[*hawN*]*G73* red-eye transformant from a generation 10 R2 subline (middle), and a non-transformed red-eye wild type individual (right).

Figure 2. *Dv*[*hawN*]*G73* transformants having a curved-wing (top) or shrivelled-wing (bottom) phenotype.

Figure 3. *In situ* hybridization of larval salivary gland chromosomes from a *Dv*[hawN]*G73* transformant hybridized with a 6.0 kb *Fsp*I *H*[w^+, hawN] restriction fragment biotinylated probe (see Fig. 4). The entire genome is shown with hybridization indicated by fluorescent staining at a single integration site (19A/B) on the X-chromosome indicated by the arrow (a). Increased magnification of the hybridization position (b), with removal of the fluorescent signal for improved band position localization (c).

adjacent 3′ *hobo* sequence (Fig. 4A). The same hybridization pattern was detected in all red-eye putative transformant samples, with no signal evident in wild-type, mutant w^{50-112}, or w^- curved-winged flies. The hybridization signals included a 4.2 kb fragment representing the mini-*white* gene, and a 6 kb fragment most likely representing 1 kb from the adjacent 3′ *hobo* arm and 5 kb of proximal insertion site chromosomal DNA. This is consistent with a single integration at the same locus in all the *Dv*[*hawN*]*G73* G1 sublines, indicating that they all arose from a single G0 integration event in line 73.

Given the ability of the autonomous HFL1 helper to integrate as well as the vector, we tested for HFL1 integration by *Fsp*I digestion (Fig. 4B). All four lines exhibited a 6.0 kb signal consistent with the *H*[w^+, hawN] *Fsp*I fragment, whereas only the R1 line exhibited, in addition, a 2.8 kb fragment consistent with the internal *Fsp*I fragment of HFL1. Further digestions and hybridizations indicated an HFL1 integration only in the R1 line (data not shown). Although HFL1 integrations could account for the curved-wing phenotypes, three sublines that have the wing phenotype do not contain HFL1. Apparently the HFL1 insertion occurred subse-

for *D. melanogaster white*$^+$ in *D. virilis* (Lozovskaya *et al.*, 1996), and *C. capitata white*$^+$ in *D. melanogaster* (Zweibel *et al.*, 1995); however, in both cases red-eye expression by single integrations was weak, leaving unresolved the possibilities for position effects or diminished gene product function. Nearly full red-eye expression in the *Dv*[*hawN*]*G73* transformant indicates for the first time that a normal *white*$^+$ phenotype can be restored by single gene interspecific complementation, and apparently can occur at least as frequently as the suppressed expression found by Lozovskaya *et al.* (1996). Too few transformants have been selected to determine whether the marker expression in *Dv*[*hawN*]*G73* was fortuitous, or whether suppression of *white*$^+$, or other markers, is actually the norm for species with abundant repetitive DNA.

hobo transformation of *D. virilis* raises the potential for use of *hobo* to transform nondrosophilid insects, yet frequencies may be considerably lower than those observed in *D. virilis*. Thus, the routine use of *hobo* may depend upon highly efficient means of transformant selection. The *white*$^+$ gene has already been cloned from *Anopheles gambiae* (Besansky *et al.*, 1995) and *C. capitata* (Zweibel *et al.*, 1995), and our results suggest that these genes, in addition to *D. melanogaster white*$^+$, may be useful as effective transformation markers in other species having *white* mutant strains. However, considering that a non-selected HFL1 integration occurred in the R1 subline, *hobo* transformation frequencies may be actually much higher than that determined solely by *white*$^+$ selection. This possibility may be resolved by a direct determination of HFL1 transformation frequency independent of marker systems.

hobo *vector stability*

Excision and transposition assays, as well as PCR experiments, indicate that cross-mobilizable or *hobo*-related systems exist in every insect species tested, including *D. virilis* (Handler & Gomez, 1995). In the present study we observed a high frequency of curved-wing phenotypes in G0s and G1 lines which were inherited, and a shrunken eye phenotype in G1s which was lethal. Although the transformants arose from inbred curved-wing G0s, they are apparently not linked and the mutations are not due to vector integration. The mutations could be due to HFL1 helper integrations, but this integration was detected in only one subline, and not in the others which also carry the wing phenotypes. Considering the potential for cross-mobilization in *D. virilis* (Handler & Gomez, 1995), it is conceivable that injected HFL1 mobilized a *hobo*-related element proximal to the mutated genes causing deletions (it is also possible, but less likely, that such elements all independently inserted into the same genes). If such interactions do occur and are common, vector stability becomes a primary concern when using *hobo* or other related vectors to create transgenic strains for mass rearing (Handler, 1993). The creation of *hobo*-mediated transformants in *D. virilis* will enable us to monitor stability after mass rearing to determine whether these concerns are valid.

Experimental procedures

Strains and rearing

The *D. virilis white*$^{50-112}$ strain (referred to as w^-) was obtained from the Bowling Green Stock Center. It exhibits a fully expressed and penetrant white eye colour phenotype, and the wild-type allele has been related to the *D. melanogaster w* locus based on sex-linkage (Alexander, 1976) and *in situ* hybridization (Lozovskaya *et al.*, 1993). Flies were reared on standard cornmeal–agar–molasses diet at 23°C.

Plasmids and injection

The *hobo* vector *H*[w^+, hawN] (Calvi *et al.*, 1991) consists of the *hobo* element in pHFL1 (Blackman *et al.*, 1989) with a mini-*white*$^+$ cassette (Pirotta *et al.*, 1985) replacing the internal *Eco*RI fragment. It is identical to *H*[w^+, haw1] except for having the single *Sal*I site in *hobo* replaced by *Not*I. The mini-*white*$^+$ gene yields an intermediate red-eye phenotype when transformed into *D. melanogaster* (Pirotta *et al.*, 1985). Transposase helper was provided by pHFL1 (HFL1) containing a complete unmodified *hobo*, or pHSH2 (HSH2) having *hobo* under *hsp70* promoter regulation (Calvi & Gelbart, 1994). Injections followed standard procedures using vector:helper concentrations of 300:100 μg/ml in injection buffer (5 mM KCl; 0.1 sodium phosphate pH 7.8).

Southern hybridization

10–20 μg of genomic DNA was digested with indicated restriction enzymes and separated on 0.8% agarose gels. DNA was stained with ethidium bromide, blotted to nylon filters and immobilized by ultraviolet irradiation. Hybridization probes were labelled with [^{32}P]dCTP by random priming (Gibco BRL) according to the manufacturer's specifications. Hybridizations were performed in phosphate buffer; 1% BSA; 7% SDS at 65°C with an initial wash in 2 × SSC; 0.2% SDS at room temperature and two washes in 1 × SSC; 0.1% SDS at 55°C for 30 min. Autoradiography was performed by exposure on Kodak X-Omat film at −90°C.

In situ *hybridization*

Salivary glands were dissected from *D. virilis* third-instar larvae in lactic acid, squashed and prepared for labelling using standard procedures. A 6.0 kb *Fsp*I fragment of *H*[w^+, hawN] containing *hobo* and mini-*white*$^+$ sequences was biotinylated by random priming (Enzo Diagnostics) and hybridized to chromosomal DNA according to Lim (1993). Hybridized probe was then reacted to streptavidin-sulforhodamine 101 (Mannheim–Boehringer) and the chromosomal DNA was labelled with 4′,6-diamidino-2-phenylindole (Sigma Chemical). Primary screening of labelled chromosomes was done by

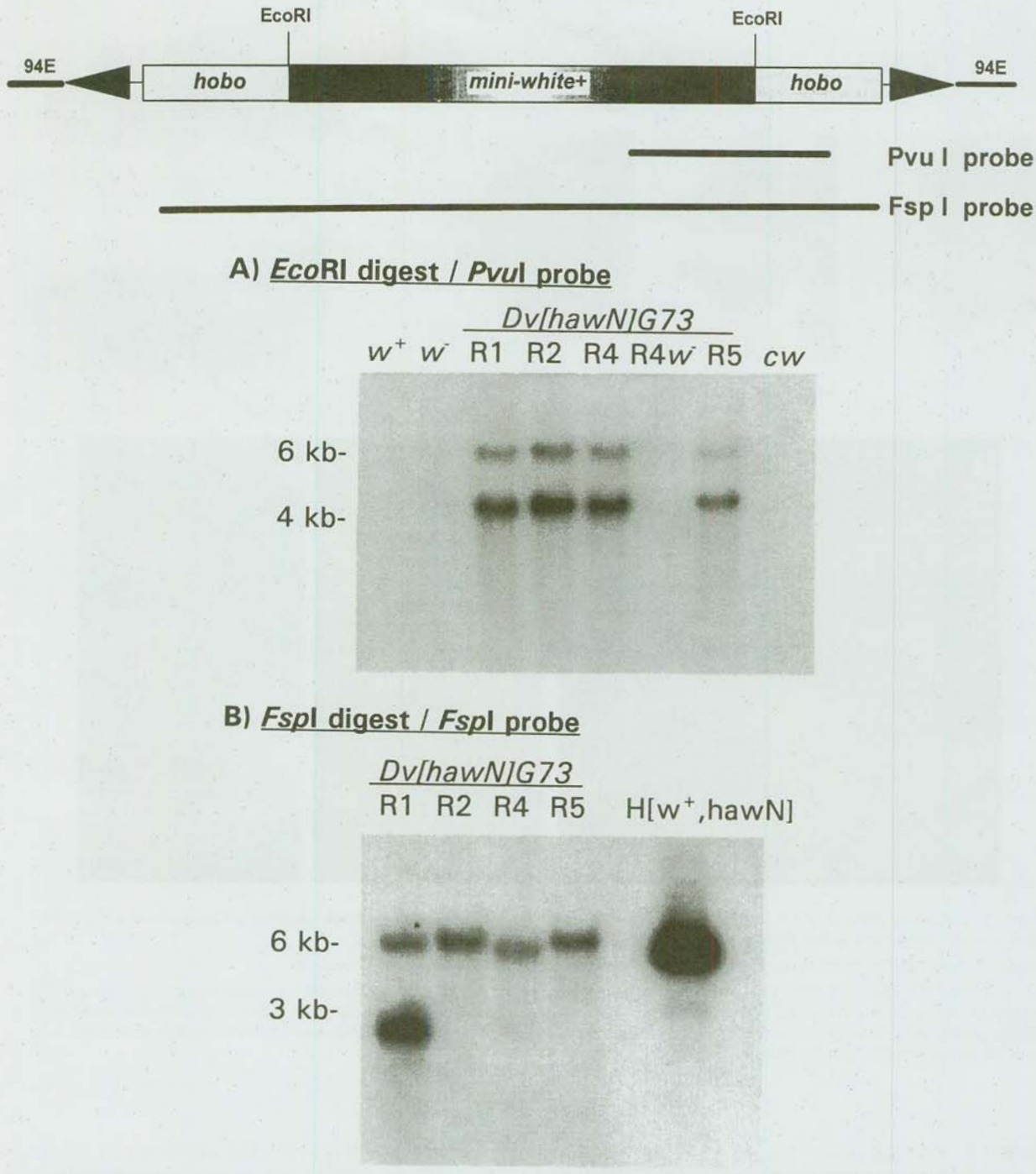

Figure 4. Schematic diagram of the $H[w^+$, hawN] vector (not to scale) containing a mini-*white*$^+$ cassette, with *Pvu*I and *Fsp*I restriction fragments used as probes for Southern hybridization and chromosomal *in situ* hybridization indicated by solid lines underneath. (A), Southern hybridization using *Pvu*I digested $H[w^+$, hawN] probe to *Eco*RI cut genomic DNA from the following samples: *D. virilis* wild-type (w^+), *white*$^{50-112}$ (w^-), *Dv*[*hawN*]*G73* transformant sublines R1, R2, R4 and R5, white-eye non-transformed R4 sibling (R4w^-), and white-eye curved-wing (*cw*). (B), Southern hybridization using *Fsp*I digested $H[w^+$, hawN] probe to *Fsp*I cut genomic DNA of indicated *Dv*[*hawN*]*G73* transformant sublines and vector plasmid.

quent to the $H[w^+$, hawN] insertion during gonadogenesis. Since the single and double insertions occurred in independent G1 lines, a total transformation frequency of 1% may be estimated from this experiment.

Direct evidence for a single chromosomal integration of $H[w^+$, hawN] is also provided by salivary gland chromosome *in situ* hybridization using the $H[w^+$, hawN] *Fsp*I biotinylated fragment as probe.

Figure 3 is representative of hybridization detected in numerous preparations from all four original transformant lines. A single insertion at 19A/B on the X-chromosome was observed consistently based on the photographic chromosome map of Gubenko & Evgen'ev (1984).

Insertion site sequence

Definitive determination of a *hobo*-mediated transposition event for $H[w^+$, hawN] is provided by the insertion site sequence of the vector–chromosome junction. An inverse polymerase chain reaction (PCR) protocol was performed in the R1 and R5 line, but yielding the same PCR product and sequence. Since the two integrations cannot be distinguished by their terminal sequences (both from *hobo*), we presume this sequence to be from the $H[w^+$, hawN] integration since HFL1 was not detected in R5. The junction sequences are given below, revealing an 8 bp direct duplication of chromosomal DNA at the insertion site (bold), which is consistent with other *hobo* and *hAT* element insertions.

CGTCAGGAA**GTGCACAC**
<**CAGAGAACT..[hawN]..AGTTCTCTG**
>**GTGCACAC**AGGCTTCA

Discussion

hobo *vector function*

The *hobo* transposable element from *D. melanogaster* is capable of mediating germline transformation in a distantly related drosophilid, *D. virilis*, catalysed by an unmodified *hobo* helper. Transformation based on marker selection occurred at a relatively low estimated frequency of 0.5% compared to an approximate 30% frequency usually achieved in *D. melanogaster* (Blackman *et al.*, 1989). Considering the additional HFL1 helper integration detected, the total transformation frequency is approximately 1%. This result is consistent with recent data from Lozovskaya *et al.* (1996), who demonstrated *hobo*-mediated transformation of *D. virilis* based on the *white*$^+$ marker at a similar frequency, but using a heat shock regulated helper. In a smaller study using HSH2 helper we failed to recover transformants, though this did not indicate a statistically different result. Thus, the prospect expressed by Lozovskaya *et al.* that a higher transformation frequency might be achieved using the endogenous *hobo* promoter is not borne out in our study. Nonetheless, this is the first demonstration of *hobo* promoter function in a species other than *D. melanogaster*.

The ability of *hobo* and related elements to retain function in a broad range of insects was first predicted by the finding that *hobo* is phylogenetically related to the plant transposons *Ac* and *Tam*3 (Calvi *et al.*, 1991), and subsequently, several insects were found to harbour transposons within the *hobo*, *Ac*, *Tam3* (*hAT*) family (Warren *et al.*, 1994; DeVault & Narang, 1994; Handler & Gomez, 1996). Of these, *Hermes* from *Musca domestica* has been found to effectively transform *D. melanogaster* despite the fact that the two species diverged ~100 Myr ago (O'Brochta *et al.*, 1996). Our confirmation of the relatively low transformation frequency of *D. virilis* by *hobo* is thus unusual considering the divergence of *D. virilis* and *D. melanogaster* ~40 Myr ago (Russo *et al.*, 1995), the similar structural relationship between *hobo* and *Hermes* (Warren *et al.*, 1994), and the similarity of *hobo* function in the two species as shown by precise excision activity (Handler & Gomez, 1995). In comparison, tephritid species diverging from *Drosophila* ~140 Myr ago (Beverley & Wilson, 1984), although also containing *hAT* transposons, are considerably less effective in supporting normal *hobo* function (Handler & Gomez, 1996).

Since an unmodified *hobo* was shown to be functional in *D. virilis* based on its *trans*-acting helper activity, as well as its own insertion, *hobo* probably functions in other drosophilid species less distantly related to *D. melanogaster* as well. It is thus interesting to consider whether the limitations on *hobo* transformation is due to factors which have restricted the presence of *hobo* to only a few subgroups of the melanogaster subgenus (Daniels *et al.*, 1990). A possibility brought forth in excision assay studies is negative interactions with *hobo*-related or *hAT* elements, or other cross-mobilizing systems which exist in many insect species (Atkinson *et al.*, 1993; Handler & Gomez, 1995, 1996). Another possibility is that the relatively low repetitive DNA content in *D. melanogaster*, 20% compared to 50% for *D. virilis* (Gall & Atherton, 1974), makes its genome more permissive for transposon integration. Thus, chromosomal transposition of *hobo* might be restricted in *D. virilis*, even though normal mobility is indicated by transient assays. On the other hand, *Hermes* transposition may be less restricted in *D. melanogaster*. The effect of repetitive DNA content on transformation may be further elucidated by testing the ability of *Hermes* to transform *D. virilis*.

Transformant selection

The low frequency of transformant recovery in *D. virilis* may also be the result of suppressed *white*$^+$ marker gene expression in the host species. This might be due to incomplete complementation between the *white*$^+$ genes in *D. melanogaster* and *D. virilis*, or a high level of position effect suppression or variegation in *D. virilis* due to its high repetitive DNA content. Interspecific complementation by *white*$^+$ genes was demonstrated

fluorescence microscopy with detailed analysis provided by high-resolution three-dimensional fluorescence microscopy (see Hiraoka *et al.*, 1991).

Polymerase chain reaction

Inverse PCR was performed by initial digestion of transformant subline genomic DNA with *Sau*3A for 5′ junctions, and *Dde*I for 3′ junctions. After 3 h digestions, restriction fragments were circularized by ligation at 12°C for 16 h. PCR was performed on the circularized fragments using primers in opposite orientation having sequences within the vector restriction site and terminus. PCR products were directly subcloned from low-melt agarose into ddT vectors (InVitrogen), which were sequenced using primers to vector sequence proximal to the respective termini.

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